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Diazotrophic Diversity and Distribution in the Tropical and Subtropical Atlantic Ocean

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To understand the structure of marine diazotrophic communities in the tropical and subtropical Atlantic Ocean, the molecular diversity of the *nifH* gene was studied by nested PCR amplification using degenerate primers, followed by cloning and sequencing. Sequences of *nifH* genes were amplified from environmental DNA samples collected during three cruises (November-December 2000, March 2002, and October-November 2002) covering an area between 0 to 28.3°N and 56.6 to 18.5°W. A total of 170 unique sequences were recovered from 18 stations and 23 depths. Samples from the November-December 2000 cruise contained both unicellular and filamentous cyanobacterial *nifH* phylotypes, as well as γ-proteobacterial and cluster III sequences, so far only reported in the Pacific Ocean. In contrast, samples from the March 2002 cruise contained only phylotypes related to the uncultured group A unicellular cyanobacteria. The October-November 2002 cruise contained both filamentous and unicellular cyanobacterial and γ-proteobacterial sequences. Several sequences were identical at the nucleotide level to previously described environmental sequences from the Pacific Ocean, including group A sequences. The data suggest a community shift from filamentous cyanobacteria in surface waters to unicellular cyanobacteria and/or heterotrophic bacteria in deeper waters. With one exception, filamentous cyanobacterial *nifH* sequences were present within temperatures ranging between 26.5 and 30°C and where nitrate was undetectable. In contrast, nonfilamentous *nifH* sequences were found throughout a broader temperature range, 15 to 30°C, more often in waters with temperature of <26°C, and were sometimes recovered from waters with detectable nitrate concentrations.

Dinitrogen (N_2) fixation is a biological process carried out by prokaryotic organisms, known as diazotrophs. These organisms are particularly important in environments where nitrogen limits primary production, as they are the only organisms capable of converting molecular N_2 into NH_4, a more readily assimilated form of dissolved nitrogen (18). Oligotrophic oceans, chronically deficient in dissolved inorganic nitrogen, should be important niches for diazotrophs (13, 18). Early work on N_2 fixation focused on the easily identifiable, abundant, filamentous cyanobacterium *Trichodesmium*, considered until recently the most important marine diazotroph (13). Imbalances in the oceanic nitrogen budget (15) prompted a reassessment of the contribution of marine diazotrophs to the oceanic nitrogen cycle (2, 5, 9, 18). Discrepancies between geochemical estimates of N_2 fixation and measured N_2 fixation rates from field populations of *Trichodesmium*, accompanied by the known biases in isolating and culturing bacteria from natural environments (25), led to the realization that the diversity and abundance of oceanic diazotrophs may have been underestimated (41).

The development of molecular methods to amplify, clone, and sequence the *nifH* gene from environmental DNA samples (40) has recently led to the discovery of new types of marine diazotrophs. The *nifH* gene, which encodes the iron protein of nitrogenase, is a highly conserved functional gene (13, 41) useful in phylogenetic studies (42). The assessment of diazotroph diversity by this approach has been performed in various environments including soils (28), freshwater and saltwater lakes (33, 39), salt marshes (4, 20), stromatolites and microbial mats (26, 31), and deep-sea vents (22). This method has yielded evidence of new, unicellular diazotrophs in both the open Atlantic and Pacific oceans (11, 12, 44, 45).

The spatial and temporal distributions of the new diazotroph phylotypes in open oceans are poorly characterized, and there is a need to assess the importance of these new groups quantitatively. Although *nifH* sequences from environmental samples are available in public databases, the success of quantitative methods such as quantitative PCR (7) and micro- or macroarrays (17, 32) is largely dependent on a good initial characterization of the diversity of the *nifH* genes in a specific study area (8).

The purpose of this study was to characterize the diversity of *nifH* in the tropical and subtropical Northern Atlantic Ocean, as well as to look for environmental factors that may influence the distribution of the various *nifH* phylotypes. Samples from surface, 1% light, and <1% light depths were analyzed and show a possible depth segregation between filamentous and unicellular cyanobacterial phylotypes. Phylogenetic relationships between *nifH* sequences recovered as part of this study and those available in GenBank were also investigated (1, 27). Several of our Atlantic *nifH* sequences were identical to environmental *nifH* sequences, which until now had only been recovered from the Pacific Ocean. This indicates that some diazotrophic phylotypes, specifically unicellular group A, may be common between the Pacific and Atlantic Oceans.

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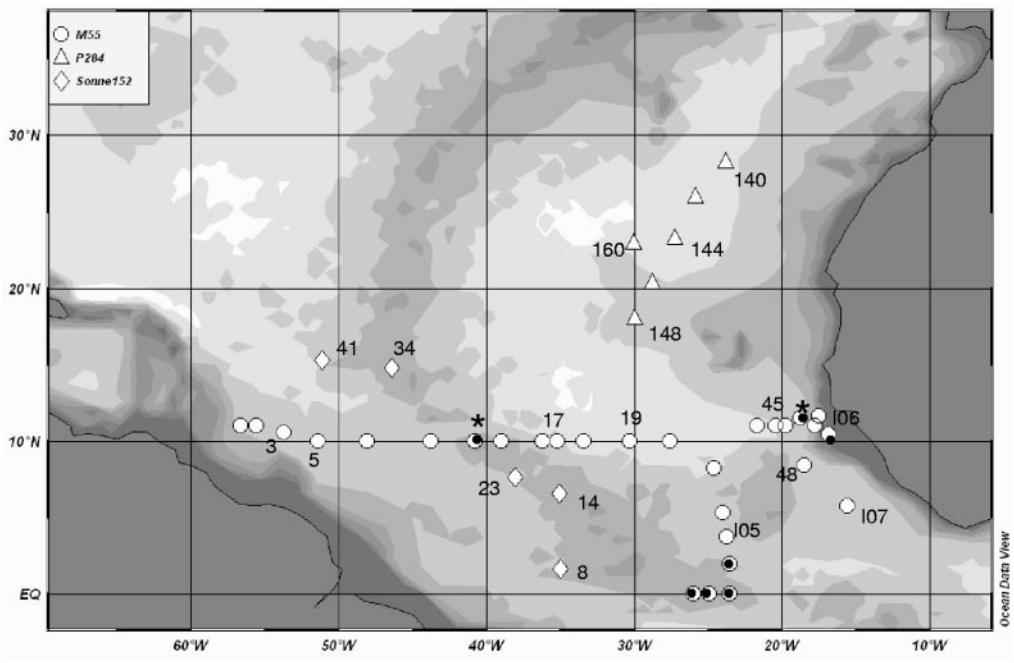

FIG. 1. Cruise tracks and stations analyzed. Stations which produced a negative *nifH* signal are indicated by a black circle within the station mark. Surface samples for stations marked with ∗ were unavailable for analysis and deepwater samples were negative. The numbered stations indicate stations from which the *nifH* amplicons were cloned and sequenced, and the numbers correspond to those listed in Table 2 and Fig. 2.

MATERIALS AND METHODS

Sample collection. Samples were collected during three cruises in the tropical and subtropical Atlantic Ocean (Fig. 1). Sonne 152 (1.7°N 35°W to 15.3°N 51.1°W) occurred during late fall 2000, where water was collected with an overboard pump from a depth of 8 m. Water from station 14 was taken from a depth of 100 m using a CTD rosette. Poseidon 284 (28.3°N 23.8°W south to 18°N 30°W north to 23°N 30°W) took place during spring 2002, and Meteor 55 (west-to-east transect along 10°N with south transect along 30°W, west along the equator, and a north transect around 23°W back to 10°N) during fall 2002. During both of these cruises, water samples were collected using a CTD rosette sampler. Three samples (I05, I06, and I07) from the Meteor 55 cruise were collected using an underway trace metal-clean diaphragm pump (1- to 3-m depth) and were used to characterize the initial diazotroph community for bioassay nutrient addition experiments (23). Water was pumped into trace metal-cleaned 60-liter carboys and siphoned into filtration bottles. All other Meteor 55 stations were sampled at three depths. A total of 28 samples from the surface (≤10 m), 26 samples from the 1% light zone, and 6 samples from depths below the 1% light level were analyzed. Samples from the Poseidon 284 cruise were collected at depths between 10 to 30 m. Seawater volumes ranging from 1.5 to 8 liters were vacuum filtered (20 to 30 kPa) through a 0.22-μm Durapore filter (Poseidon and Meteor) or through a 0.2-μm Isopore filter (Sonne 152). All filters were stored at −80°C in cryovials until nucleic acid extraction in the laboratory. Samples for nucleic acid extraction were not prescreened to remove net plankton to study the entire diazotrophic community. Samples for nutrient analysis were collected using the CTD rosette sampler and analyzed on board (14). Nutrient data were available for all three cruises; however, the data for the Sonne 152 cruise had very low resolution in the upper 500 m and could not be used for our study. Cruise track coordinates were graphed with Ocean Data View (29).

DNA amplification and sequence analysis. DNA was extracted using the QIAGEN DNeasy Plant mini kit. All extracted DNA samples were checked for integrity by agarose gel electrophoresis. In all cases, bands of large sizes were obtained, which indicated that the DNA was of high quality and not degraded. *nifH* sequences were amplified from environmental DNA (1 to 20 ng/μl) using Taq-Gold polymerase (Applied Biosystems); the primers and nested PCR method are described in reference 39, with the following modifications. The first round of PCR (10× buffer II, $MgCl_2$ [final concentration, 4 mM], 10 mM deoxynucleoside triphosphates, 80 pmol of each primer [nifH3 and nifH4]), consisted of 10 min at 95°C; 35 cycles, each consisting of 1 min at 95°C, 1 min at 45°C, and 1 min at 72°C; and finally 10 min at 72°C. The second round (10× buffer II, $MgCl_2$ [final concentration, 6 mM], 10 mM deoxynucleoside triphosphates, 80 pmol of each primer [nifH1 and nifH2]) used a similar thermocycler program but with an annealing temperature of 54°C and only 28 cycles. Amplicons of the predicted 359-bp size, as proven by gel electrophoresis, were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Randomly picked clones were sequenced in the reverse direction using the T7 primer. Sequences were placed in the correct reading frame and trimmed to an approximately 345-bp segment corresponding to bases 639 to 984 in *Azotobacter vinelandii* with BioEdit Sequence Alignment Editor (16). Alignments were performed with ClustalX, version 1.6 (34). Sequence names were indicated as follows: TA, tropical Atlantic; P, S, or M, first letter of the ship on which the sample was collected; sample number; and clone number.

A *nifH* sequence database for phylogenetic analysis was assembled by combining the recovered sequences with *nifH* sequences (11, 42) from GenBank. This included sequences that had the highest similarity to the recovered sequences, as determined through BLASTx and BLASTn searches, and sequences obtained from other environmental *nifH* studies. This database, used for all phylogenetic analyses, contained >300 sequences, trimmed to the same segment and length as our recovered sequences. Our sequences and those from the database were analyzed for frameshifts by translation into amino acid sequences in all six frames. Sequences containing frameshifts were excluded from the analysis. Groups of identical sequences were represented by only one sequence.

TABLE 1. Temperature and nutrient concentrations for the three North Atlantic cruises[a]

Cruise	Date (mo yr)	Temperature (°C)		NO_3 (μM)	
		Mean	SD	Mean	SD
Sonne 152	Oct. 2000	26.7	1.0	NA	NA
Poseidon 284	Apr. 2002	23.0*	1.3	0.01	0.01
Meteor 55-surface	Nov. 2002	28.5	0.6	0.09	0.18
Meteor 55-deep	Nov. 2002	19.9*	3.1	9.02	9.85

[a] All temperatures were significantly different from one another except for the two sample sets indicated by asterisks. NA, not available.

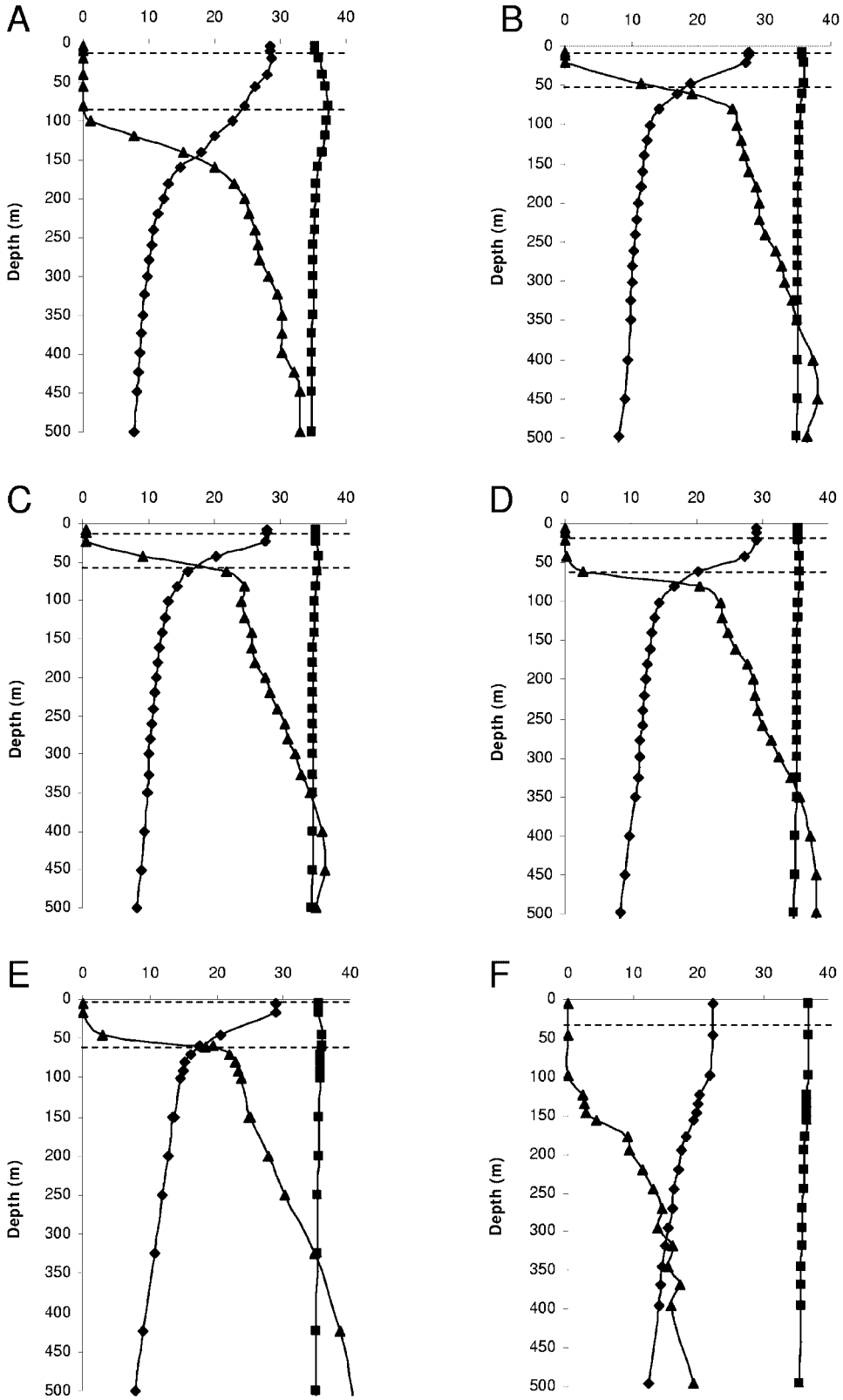

FIG. 2. Representative vertical profiles for temperature, salinity, and nitrate during the Meteor 55 and Poseidon cruises. All profiles show depth (in meters) on the *y* axis. The following variables are plotted on the same scale on the *x* axis as follows: ◆, temperature in degrees Celsius; ■, salinity in parts per thousand; and ▲, NO_3 in micromoles per liter. The dashed lines mark the depths from which samples were collected. Station numbers correspond to those given in Table 2 and Fig. 1: Meteor station 3, which is influenced by the Amazon Plume (A), Meteor station 17 (B), Meteor station 19 (C), Meteor station 45 (D), Meteor station 48 (E), and Poseidon station 144 (F) (representative of all Poseidon stations).

TABLE 2. Summary of station and cloning information[a]

Sta	Pos	Depth (m)	Sample no.	No. of clones	Cluster I									Cluster III
					Unicellular cyanobacterium			Filamentous cyanobacterium			γ-Proteobacterium			
					Cy	Cr	UA	Tt	Te	Ks	Kl	AO	PO	
8	1.7°N, 35.0°W	8	TAS8**	10									100	
14	6.6°N, 35.1°W	100	TAS14**	11	9.1			45.5						45.5
23	7.6°N, 38.0°W	8	TAS23**	24				66.7		33.3				
34	14.8°N, 46.4°W	8	TAS34**	14			100							
41	15.3°N, 51.1°W	8	TAS41**	9		44		33.3			11.1			11.1
140	28.3°N, 23.8°W	10	TAP140**	11			100							
144	23.13°N, 27.3°W	30	TAP144**	8			100							
148	18.0°N, 30.0°W	20	TAP148**	11			100							
160	23.0°N, 30.0°W	20	TAP160**	11			100							
3	10.6°N, 53.7°W	5	TAM0128	11				81.8	9.1	9.1				
3	10.6°N, 53.7°W	80	TAM0123**	15			100							
5	10.0°N, 51.4°W	4	TAM0228**	14				50		28.6		21.4		
17	10.0°N, 33.4°W	8	TAM0797**	11				36.4		63.6				
17	10.0°N, 33.4°W	47	TAM0794**	25				48	4		20	28		
19	10.0°N, 30.3°W	8	TAM0902**	19				68.4		31.8				
19	10.0°N, 30.3°W	62	TAM0898**	12	100									
I05	3.7°N, 23.7°W	<5	TAM105**	39		2.6		30.8		66.7				
I06	11°N, 17.8°W	<5	TAM106**	26			3.8	11.5	3.8	80.8				
45	11°N, 20.4°W	6	TAM2179**	13				84.6		15.4				
45	11°N, 20.4°W	61	TAM2175**	24			100							
I07	5.8°N, 15.6°W	<5	TAM107**	25				52		48				
48	8.4°N, 18.5°W	7	TAM2305**	8					100					
48	8.4°N, 18.5°W	62	TAM2294**	6							16.7	83.3		
Total no. of recovered sequences				354	13	5	95	108	11	87	6	15	111	6
Total no. of unique sequences				170	5	2	34	58	8	38	6	10	6	2

[a] The station numbers and positions correspond to those in Fig 1. Sequences are grouped by station (Sta) and cruise (TAS-Sonne, TAP-Poseidon, and TAM-Meteor). The sample collection depths in meters (Depth m) and station positions (Pos) are given. **, clone number. No. of clones, number of clones used for phylogenetic analyses from each station. The columns following correspond to the percentage of the recovered sequences with high similarity to cluster I *nifH Cyanothece* (Cy), *Crocosphaera*/*Synechocystis* (Cr), unicellular group A (UA), *T. thiebautii* (Tt), *T. erythraeum* (Te), *K. spiralis* (Ks), *Klebsiella pneumoniae* (Kl), an environmental clone from the Atlantic Ocean (AO), an environmental clone from the Pacific Ocean (PO), or cluster III *nifH*.

Phylogenetic analysis was performed using TREECON (36). Distances were estimated by the Kimura method ($P/Q = 2$ for nucleotide analyses), and the neighbor-joining method was utilized for inferring tree topologies. Both nucleotide and protein trees were constructed several times as in reference 43 with the *nifH* database, changing the outgroup organisms and bootstrap calculations. Sequences for a protochlorophyllide reductase enzyme from *Leptolyngbya boryana* (gi:441179), a nitrogenase subunit *NifH* ATPase from *Trichodesmium erythraeum* ISM101 (gi:23042334), and *Methanobrevibacter arboriphilicus* (gi:780707) were tried as outgroups for the protein tree without causing major changes to the clades. The *nifH* sequence from *Archea* member *Methanosarcina barkerii* (AB019139.1) was used as the outgroup as previously described (11).

Nucleotide sequence accession numbers. The environmental sequences recovered from this study have been placed in the GenBank database with accession numbers AY896295 through AY896469.

RESULTS

Diazotrophic distribution and environmental conditions. As indicated by near detection limit or undetectable surface water nutrient concentrations, all samples were collected in oligotrophic waters (38), with the exception of three samples from below the mixed layer, in which NO_3 concentrations were elevated, reaching values as high as 21.9 μM (Table 1). In contrast, water temperatures differed significantly during the three cruises. Warmest temperatures were observed in surface waters during the Meteor 55 west-east transect (Table 1). The coldest temperatures were recorded in surface waters during the Poseidon cruise at latitudes between 18 and 28°N, as well as in deepwater samples. Mixed layer depths during the Meteor 55 cruise ranged between 20 and 30 m for the east-west transect and around 50 m at the equator (Fig. 2A to E) (10). Deep mixed layer depths (50 to 70 m), in addition to poor thermal stratification, were characteristic throughout all Poseidon stations, which may explain the lower surface water temperature there (Fig. 2F).

The target *nifH* segment was recovered nearly everywhere throughout the area studied, except for along the equator and at a few stations near the African coast (Fig. 1). The distribution of phylotypes found at selected stations is presented in Table 2. In cloned samples, *nifH* sequences from *Trichodesmium* spp. were present throughout much of the Atlantic Ocean surface waters at latitudes ranging from 4 to 16°N, as previously reported for this area (15). However, a shift to *nifH* sequences clustering with unicellular cyanobacteria and γ-proteobacterial phylotypes was detected in water samples collected below a depth of 10 m (Table 2). Group A *nifH* sequences were the only types recovered during the Poseidon cruise (18 to 28°N). In addition, this phylotype was only recovered once from samples where *Trichodesmium* sequences were dominant. γ-Proteobacterial sequences were not as common as cyanobacterial *nifH* sequences and had a scattered distribution throughout the sample area. Cluster III *nifH* sequences were

uncommon in our sample set and found only twice in samples collected from the western and central tropical Atlantic Ocean.

***nifH* Diversity and phylogenetic analysis.** Overal, 354 *nifH* sequences were recovered from 19 stations and 23 depths during the three cruises. Of these recovered sequences, 170 were unique and fell into clusters I and III of the previously described *nifH* gene clusters (Fig. 3) (22, 42). Only 2% of the recovered *nifH* sequences grouped within cluster III; a group containing phototrophic, anaerobic bacteria, and sulfate reducers such as chlorobi, δ-proteobacteria, and spirochaetes. The two unique *nifH* nucleic acid sequences recovered in our study showed 99% similarity to another cluster III environmental *nifH* sequence (Fig. 4) previously isolated from the Hawaii ocean time series station in the Pacific Ocean (7). Sequences within this clade are all derived from uncultured marine bacteria that have approximately 83% similarity to the *nifH* sequence from *Chlorobium tepidum*, the closest identified bacterium.

The rest of the *nifH* sequences clustered either with the cyanobacterial (Fig. 5) or γ-proteobacterial (Fig. 6) clades of cluster I. Thirty-two percent of the recovered sequences had high similarities to unicellular cyanobacterial phylotypes and grouped with other environmental sequences to form three distinct clades (Fig. 5): the unicellular group A clade, consisting of uncultured environmental sequences distantly related to *Cyanothece*; the *Cyanothece*-like clade, with environmental sequences closely related to the genus *Cyanothece*; and the unicellular group B, represented by *Crocosphaera watsonii.* Almost all (84%) of our unicellular sequences clustered with group A phylotypes. The group A *nifH* sequence was originally characterized in samples collected at the HOT station. In our analysis, the group A cluster was interspersed with sequences from both the Pacific and Atlantic Oceans. Furthermore, nucleic acid sequences, which were recovered several times from seven of our Atlantic sites, were identical to the Pacific clones HT1150 (AF059627.1) and HT1205 (AF059642.1), suggesting that some unicellular cyanobacterial phylotypes may be cosmopolitan in tropical oceans. Similar results were obtained for the group B clade.

Filamentous cyanobacterial *nifH* phylotypes were abundant in our clone libraries and appeared to be less divergent than unicellular cyanobacterial sequences. The known species, *Trichodesmium thiebautii*, *T. erythraeum*, and *Katagnymene spiralis*, did not form three separate clades but clustered into two clades, with *T. erythraeum* forming a clade alone. Sequences from *K. spiralis* and *T. erythraeum* are identical at the amino acid level, but at the nucleotide level, *K. spiralis* and *T. thiebautii* appear to be more closely related.

The remaining cluster I sequences (9%) had the highest similarity to γ-proteobacteria (Fig. 6). These sequences separated into two distantly related clades, which were <80% homologous to each other. These clades were composed solely of environmental sequences from uncharacterized, uncultured organisms but were distantly related to *Vibrio diazotrophicus*. Sequences within the first clade were >97% similar to an environmentally recovered clone, AO1113 (AAC36068.1), originally described in the Atlantic Ocean but with representatives in the Pacific Ocean. The second clade was only distantly related (87 to 90% similarity) to the previously isolated Pacific Ocean clone PO3137 (AF059647.1).

Overall, 11 different *nifH* phylotypes were recovered from our study of the tropical Atlantic. Graphs of phylotype occurrence as a function of temperature and depth or nitrate concentration suggest that the pattern of species distribution may be highly influenced by temperature (Fig. 7). With one exception, filamentous cyanobacteria and *Crocosphaera* sequences were found at a temperature range of 27 to 30°C and in waters with undetectable nitrate concentrations. In contrast, group A and *Cyanothece*-like phylotypes were present over a wide temperature range, from 16 to 29°C. Similarly, most *nifH* phylotypes were detected in nitrate-depleted waters, but some group

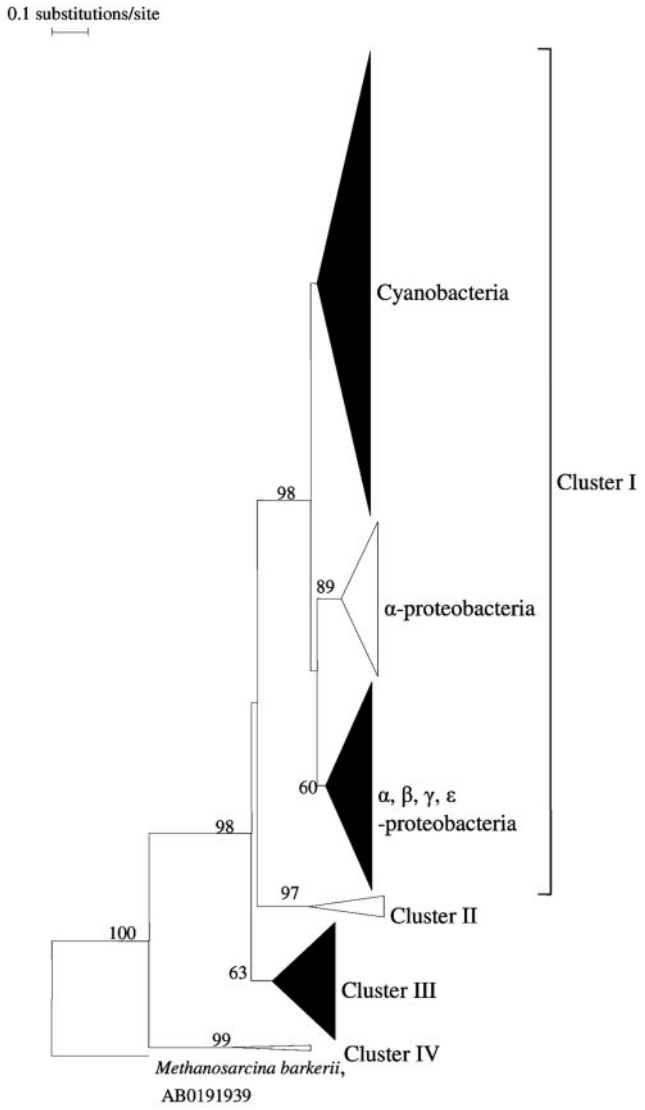

FIG. 3. Phylogenetic tree of global NifH protein sequences. To better visualize the relationship between the groups, only the major clusters and clades with significant bootstrap values (>60) were shown. Sizes of the triangles reflect the number of sequences in that clade. Clades that contained sequences from our study are shown in black. Cluster I includes cyanobacteria, α-proteobacteria, β-proteobacteria, γ-proteobacteria, and ε-proteobacteria. Cluster II consists of δ-proteobacteria, spirochaetes, and members of the *Archeaea*. Members of the *Firmicutes*, *Spirochaeta*, δ-proteobacteria, chlorobi, and *Archaea* comprise cluster III. Cluster IV is formed by archaeal sequences. The tree was bootstrapped 100 times with *Methanosarcina barkerii* AB0191939 as the outgroup.

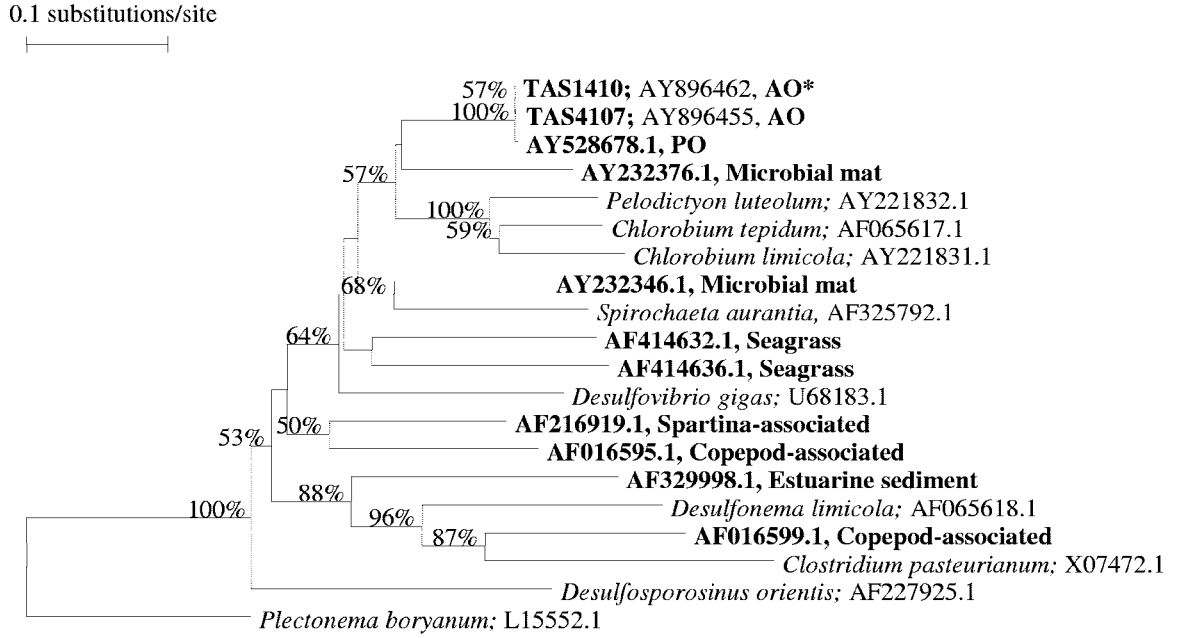

FIG. 4. Phylogenetic tree of cluster III *nifH*. Environmental sequences are shown in boldface type, followed by the environment from where they were collected. AO, Atlantic Ocean; PO, Pacific Ocean. AY896462, marked with an asterisk, represents five sequences of 100% similarity at the nucleotide level. Relationships were bootstrapped 1,000 times, and values above 50% are shown. This tree is based on Fig. 3b in reference 8.

A phylotypes were also observed where nitrate concentrations were high. The depth segregation of phylotypes is noticeable in the data shown in Table 2. In all four stations where surface and deep samples were analyzed, filamentous cyanobacterial *nifH* sequences were restricted to surface waters, and unicellular cyanobacterial or γ-proteobacterial sequences dominated deepwater clone libraries.

DISCUSSION

The results from our study document the presence and diversity of *nifH* phylotypes in the tropical and subtropical Atlantic. As expected, *nifH* sequences were found throughout the surface water samples of the tropical Atlantic, except for samples collected at the equator and at some upwelling stations near the African coast (6, 35). Parallel measurements of depth-integrated N_2 fixation rates during the Meteor 55 cruise varied from 3.7 to 255 μmol N m^{-2} day^{-1}, with the lowest rates at the equator (37). This supports the observed absence of *nifH* sequences in our nucleic acid samples from equatorial stations.

Filamentous cyanobacterial *nifH* phylotypes were recovered mainly from warm surface waters, and many features of the ecophysiology of *Trichodesmium* may explain this distribution pattern (for a review, see reference 19). The distribution of *Trichodesmium* is mostly limited to seawater temperatures of >20°C, but other factors correlated with high temperature, such as high levels of light and low levels of nutrients, may also be partly responsible for the observed temperature dependence (18). Laboratory experiments have corroborated that the optimal growth temperature range for pure cultures of *Trichodesmium* is between 25 and 30°C (3) and that high water temperatures may be required for effective nitrogenase activity in this nonheterocystous cyanobacteria (30). Input of iron through atmospheric dust deposition is another possible determinant of *Trichodesmium* distribution. The equatorial stations had low levels of dissolved iron (10) at the time of sampling; this may explain why filamentous diazotrophs, considered to have a high iron requirement (19), were not recovered in samples from this oligotrophic, upwelling region. Two complementary studies on Meteor 55 concluded that iron was an important factor affecting the activity of diazotrophs in the tropical Atlantic (23, 37). Voss et al. (37) found a significant correlation between depth-integrated total dissolved iron and depth-integrated N_2 fixation levels. Mills et al. (23) determined that even at the easternmost stations, addition of dissolved iron or of iron-rich Saharan dust stimulated N_2 fixation relative to control incubations.

The relative abundance of *nifH* phylotypes in our clone libraries suggests a segregation of filamentous sequences at the surface and unicellular cyanobacterial and heterotrophic bacterial sequences in deeper water. However, the abundance of specific phylotypes in a clone library is semiquantitative at best and must be interpreted with caution. The results we obtained from the clone libraries will need to be confirmed by more quantitative methods that account for possible PCR and cloning biases. Nevertheless, the predominance of *Trichodesmium* phylotypes in surface waters and lack thereof in deeper waters fit well with the autecology of *Trichodesmium.* Deepwater samples were collected at temperatures below the optimal growth range (25 to 30°C) of filamentous cyanobacteria, which may be one explanation for this observation. In addition, the presence of intracellular gas vacuoles, which give buoyancy to *Trichodesmium* (18), could also contribute to the apparent dominance of the filamentous phylotypes in surface waters. The dominance of *Trichodesmium* in surface waters of the tropical North Atlantic (35) and the observed dominance of unicellular cya-

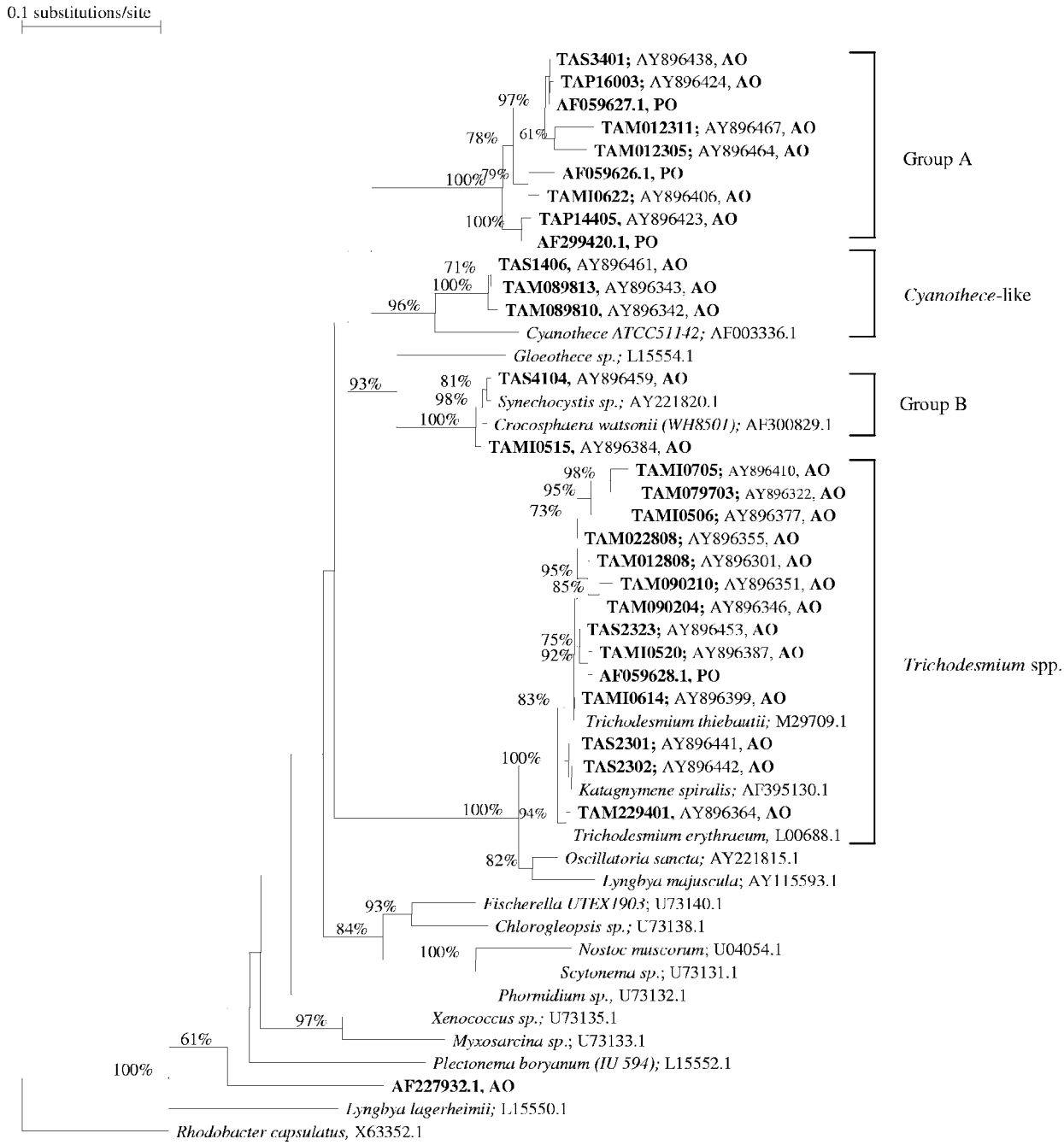

FIG. 5. Phylogenetic relationships of *nifH* cyanobacterial sequences. Environmental sequences are shown in boldface type, followed by the environment from where they were collected. AO, Atlantic Ocean; PO, Pacific Ocean. Relationships were bootstrapped 1,000 times, and values over 50% were shown, except for those in the *Trichodesmium* spp. group where values above 70% are shown. For visual purposes, only environmental sequences which had bootstrap values above 70% are shown.

nobacterial *nifH* phylotypes throughout the mixed layer in the Pacific Ocean (24) also support our observations.

Although the occurrence of *Crocosphaera nifH* phylotypes was restricted to a temperature range comparable to that of *Trichodesmium*, two other unicellular cyanobacterial phylotypes were found at temperatures as low as 15°C. Group A sequences were the only phylotypes recovered from several stations located between 18 and 28°N, where the mean surface water temperature was 23°C. In contrast, Mazard et al. (21) found that unicellular cyanobacterial diazotrophs were restricted to temperatures above 25°C, similar to filamentous cyanobacteria distributions. This discrepancy may be due to the fact that their study did not include the uncultured group A phylotype, which is uncharacterized by a 16S rRNA sequence. Whether or not the organisms present at these low temperatures and depths actively fix nitrogen can only be resolved with further studies by methods, such as reverse

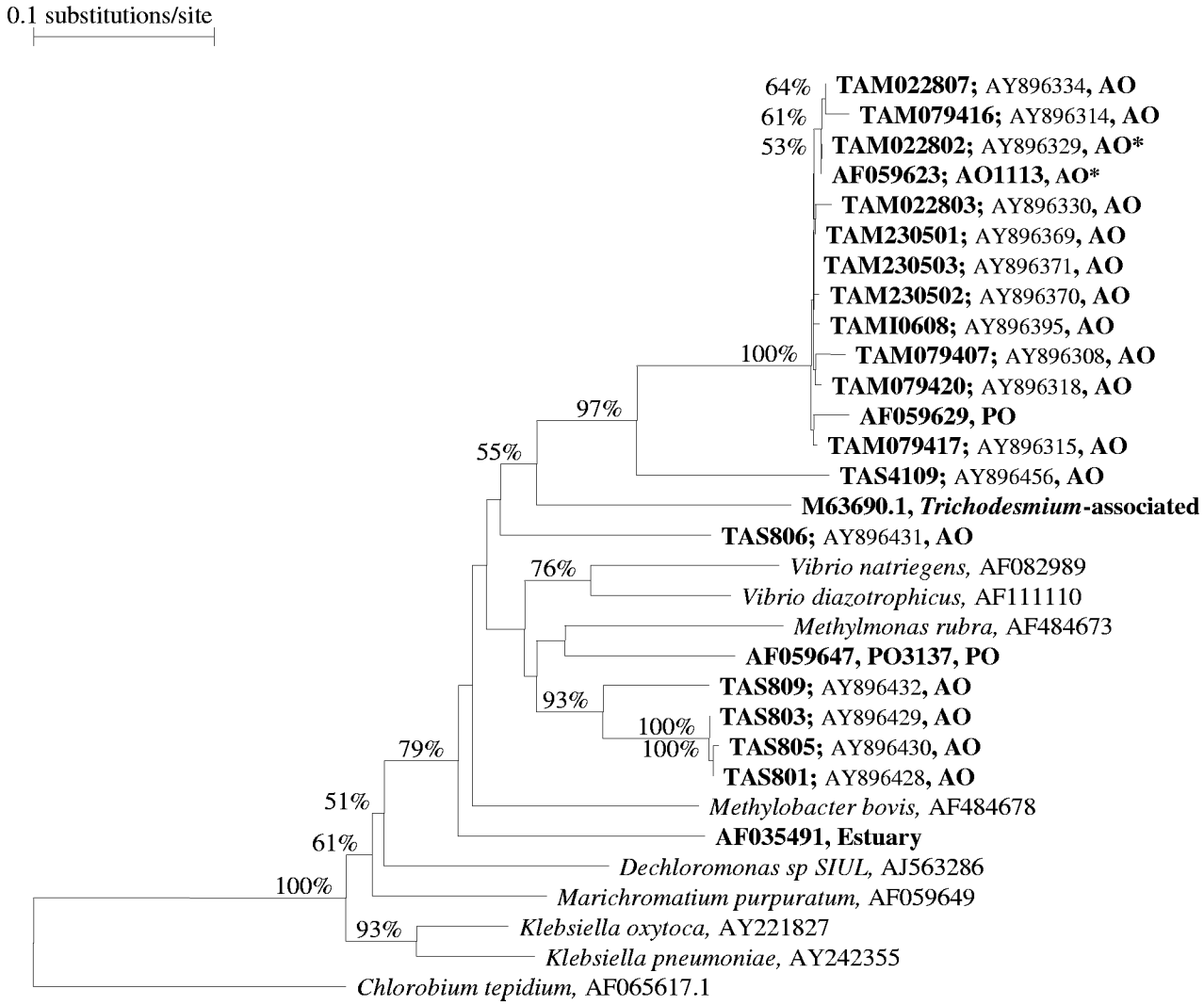

FIG. 6. Phylogenetic relationships between γ-proteobacteria *nifH* nucleotide sequences. Sequence names in boldface type indicate environmental sequences and are marked according to recovery from the Pacific Ocean (PO) or the Atlantic Ocean (AO). Sequence names beginning with "TA" were obtained in this study. AY896329 and AF059623, indicated by asterisks, represent several sequences which were identical. The tree was bootstrapped 1,000 times, and values over 50% are shown.

transcription-PCR, that allow the monitoring of RNA expression. However, Voss et al. (37) observed a trend of high N_2 fixation at the surface, which rapidly decreased with depth. One exception was at eastern stations, where rates of 3.1 nmol N liter^{-1} h^{-1} at the surface were comparable to rates of 2.2 nmol N liter^{-1} h^{-1} from below the mixed layer and where high nitrate concentrations prevailed. Since both the group A and the γ-proteobacteria phylotypes were recovered in this area, they are potential candidates for diazotrophic activity in the deeper waters.

It has been suggested that unicellular cyanobacteria populations from the Atlantic and Pacific Oceans are divergent (11). In contrast, this study found several phylotypes, especially group A, which had 100% homology at the nucleic acid level with sequences found in the Pacific, indicating that some diazotroph phylotypes are common to both oceans. A total of 66 sequences were found to be identical at the nucleotide level with sequences isolated from the Pacific Ocean, and many others with 99% similarity formed clades with Pacific Ocean sequences. Furthermore, our results also demonstrated a very high degree of similarity (99%) between two cluster III *nifH* sequences from the Pacific Ocean and ours from the Atlantic. This is the first time that this cluster III sequence has been reported for Atlantic samples. As we have not collected samples in the Pacific Ocean, potential DNA contamination of the samples can be ruled out. The high conservation between Pacific and Atlantic phylotypes will be useful when designing nucleic acid primers and probes for the enumeration of *nifH* phylotypes. N_2 fixation in the Atlantic Ocean appears to be potentially performed by a few species that have widespread distribution, based on *nifH* gene DNA analyses.

This study has presented data on the potential for N_2 fixation through the analysis of *nifH* gene diversity across the Atlantic Ocean. Our data suggest a potential community shift from filamentous cyanobacterial phylotypes to unicellular cyanobacterial or heterotrophic bacterial phylotypes with depth, and we hypothesize that growth temperature preferences of

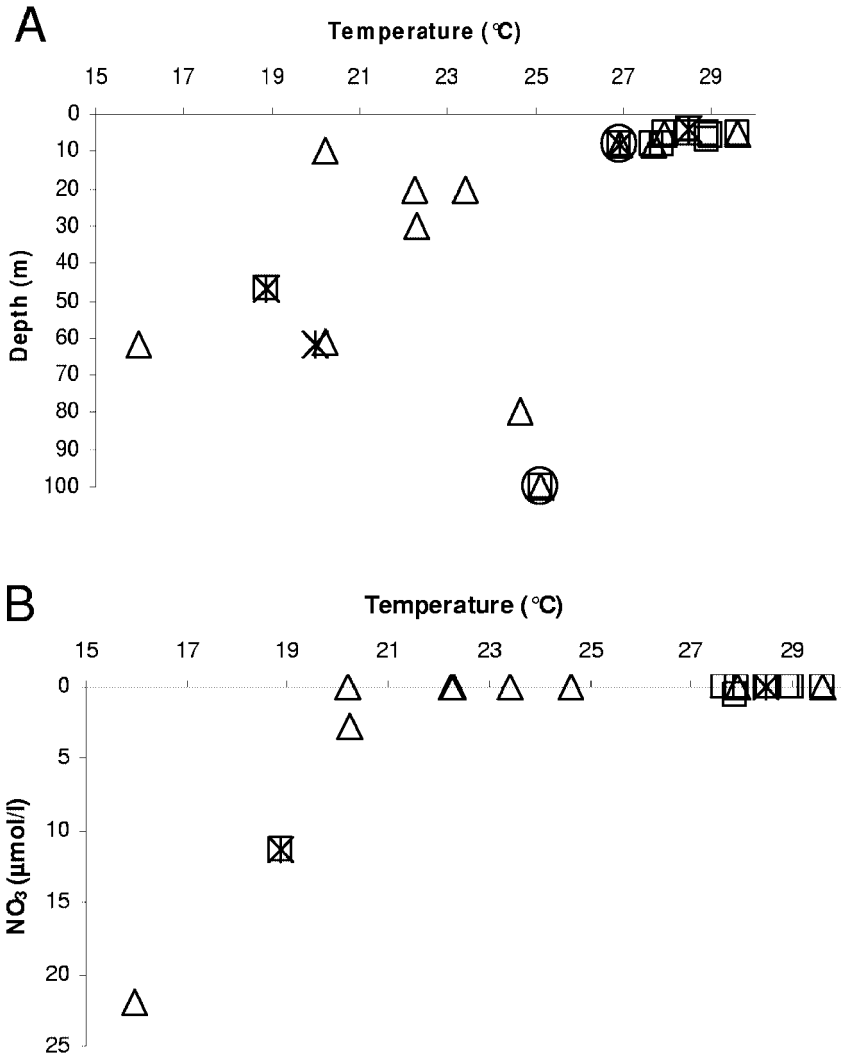

FIG. 7. Phylotype occurrence. □, filamentous cyanobacteria; Δ, unicellular cyanobacteria; ∗, γ-proteobacteria; ○, cluster III bacteria graphed as a function of sample temperature versus sample depth (in meters) (A) or nitrate concentration (in micromoles per liter) (B). There are fewer points in panel B because nutrient data were not available from the Sonne cruise (see Materials and Methods).

the various diazotrophs may explain the observed differences. While group A diazotrophs have already been shown to be important and widespread in the Pacific Ocean, this study revealed that they are also widely distributed throughout the Atlantic Ocean.

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REFERENCES

1. **Allen, A. E., M. G. Booth, M. E. Frischer, P. G. Verity, J. P. Zehr, and S. Zani.** 2001. Diversity and detection of nitrate assimilation genes in marine bacteria. Appl. Environ. Microbiol. **67:**5343–5348.
2. **Berman-Frank, I., P. Lundgren, and P. Falkowski.** 2003. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Res. Microbiol. **154:** 157–164.
3. **Breitbarth, E.** 2005. Ecophysiology of the cyanobacterium *Trichodesmium*. Ph.D. thesis. Christian Albrechts University of Kiel, Kiel, Germany.
4. **Brown, M. M., M. J. Friez, and C. R. Lovell.** 2003. Expression of nifH genes by diazotrophic bacteria in the rhizosphere of short form Spartina alterniflora. FEMS Microbiol. Ecol. **43:**411–417.
5. **Capone, D. G., J. P. Zehr, H. W. Paerl, B. Bergman, and E. J. Carpenter.** 1997. *Trichodesmium*, a globally significant marine cyanobacterium. Science **276:**1221–1229.
6. **Carpenter, E. J., A. Subramaniam, and D. G. Capone.** 2004. Biomass and primary productivity of the cyanobacterium *Trichodesmium* spp. in the tropical N Atlantic ocean. Deep Sea Res. I Oceanogr. Res. Pap. **51:**173–203.
7. **Church, M. J., B. D. Jenkins, D. M. Karl, and J. P. Zehr.** 2005. Vertical distributions of nitrogen-fixing phylotypes at Stn ALOHA in the oligotrophic North Pacific Ocean. Aquat. Microb. Ecol. **38:**3–14.
8. **Church, M. J., C. M. Short, B. D. Jenkins, D. M. Karl, and J. P. Zehr.** 2005. Temporal patterns of nitrogenase (*nifH*) gene expression in the oligotrophic North Pacific Ocean. Appl. Environ. Microbiol. **71:**5362–5370.
9. **Codispoti, L. A.** 1997. The limits to growth. Nature **387:**237–238.
10. **Croot, P. L., P. Streu, and A. R. Baker.** 2004. Short residence time for iron in surface seawater impacted by atmospheric dry deposition from Saharan dust events. Geophys. Res. Lett. **31**. [Online.] doi:10.1029/2004GL020153.
11. **Falcon, L. I., E. J. Carpenter, F. Cipriano, B. Bergman, and D. Capone.** 2004.

N2 fixation by unicellular bacterioplankton from the Atlantic and Pacific Oceans: phylogeny and in situ rates. Appl. Environ. Microbiol. **70:**765–770.
12. **Falcon, L. I., F. Cipriano, A. Y. Chistoserdov, and E. J. Carpenter.** 2002. Diversity of diazotrophic unicellular cyanobacteria in the tropical North Atlantic Ocean. Appl. Environ. Microbiol. **68:**5760–5764.
13. **Falkowski, P. G.** 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. Nature **387:**272–275.
14. **Grasshoff, K., M. Ehrhardt, and K. Kremling.** 1983. Methods of seawater analysis. Springer-Verlag, New York, N.Y.
15. **Gruber, N., and J. L. Sarmiento.** 1997. Global patterns of marine nitrogen fixation and denitrification. Global Biogeochem. Cycles **11:**235–266.
16. **Hall, T.** 2004. BioEdit, version 6.0.7. Ibis Therapeutics, Carlsbad, Calif.
17. **Jenkins, B. D., G. F. Steward, S. M. Short, B. B. Ward, and J. P. Zehr.** 2004. Fingerprinting diazotroph communities in the Chesapeake Bay by using a DNA macroarray. Appl. Environ. Microbiol. **70:**1767–1776.
18. **Karl, D., A. Michaels, B. Bergman, D. Capone, E. Carpenter, R. Letelier, F. Lipschultz, H. Paerl, D. Sigman, and L. Stal.** 2002. Dinitrogen fixation in the world's oceans. Biogeochemistry **57:**47–98.
19. **La Roche, J., and E. Breitbarth.** 2005. Importance of the diazotrophs as a source of new nitrogen in the ocean. J. Sea Res. **53:**67–91.
20. **Lovell, C. R., M. J. Friez, J. W. Longshore, and C. E. Bagwell.** 2001. Recovery and phylogenetic analysis of *nifH* sequences from diazotrophic bacteria associated with dead aboveground biomass of *Spartina alterniflora*. Appl. Environ. Microbiol. **67:**5308–5314.
21. **Mazard, S. L., N. J. Fuller, K. M. Orcutt, O. Bridle, and D. J. Scanlan.** 2004. PCR analysis of the distribution of unicellular cyanobacterial diazotrophs in the Arabian Sea. Appl. Environ. Microbiol. **70:**7355–7364.
22. **Mehta, M. P., D. A. Butterfield, and J. A. Baross.** 2003. Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. Appl. Environ. Microbiol. **69:**960–970.
23. **Mills, M. M., C. Ridame, M. Davey, J. La Roche, and R. J. Geider.** 2004. Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. Nature **429:**292–294.
24. **Montoya, J. P., C. M. Holl, J. P. Zehr, A. Hansen, T. A. Villareal, and D. Capone.** 2004. High rates of N_2 fixation by unicellular diazotrohps in the oligotrophic Pacific Ocean. Nature **430:**1027–1032.
25. **Nuebel, U., F. Garcia-Pichel, M. Kuehl, and G. Muyzer.** 1999. Quantifying microbial diversity; morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. Appl. Environ. Microbiol. **65:**422–430.
26. **Omoregie, E. O., L. L. Crumbliss, B. M. Bebout, and J. P. Zehr.** 2004. Determination of nitrogen-fixing phylotypes in *Lyngbya* sp. and *Microcoleus chthonoplastes* cyanobacterial mats from Guerrero Negro, Baja California, Mexico. Appl. Environ. Microbiol. **70:**2119–2128.
27. **Prieme, A., G. Braker, and J. M. Tiedje.** 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. Appl. Environ. Microbiol. **68:**1893–1900.
28. **Rosado, A. S., G. F. Duarte, L. Seldin, and J. D. Van Elsas.** 1998. Genetic diversity of *nifH* gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR-amplified gene fragments. Appl. Environ. Microbiol. **64:**2770–2779.
29. **Schlitzer, R.** 2004. Ocean Data View, 2.0 ed. Alfred Wegener Institute, Bremerhaven, Germany.
30. **Staal, M., F. J. R. Meysman, and L. J. Stal.** 2003. Temperature excludes N2-fixing heterocystous cyanobacteria in the tropical oceans. Nature **425:** 504–507.
31. **Steppe, T. F., J. L. Pinckney, J. Dyble, and H. W. Paerl.** 2001. Diazotrophy in modern marine Bahamian stromatolites. Microb. Ecol. **41:**36–44.
32. **Steward, G. F., B. D. Jenkins, B. B. Ward, and J. P. Zehr.** 2004. Development and testing of a DNA macroarray to assess nitrogenase (*nifH*) gene diversity. Appl. Environ. Microbiol. **70:**1455–1465.
33. **Steward, G. F., J. P. Zehr, R. Jellison, J. P. Montoya, and J. T. Hollibaugh.** 2004. Vertical distribution of nitrogen-fixing phylotypes in a meromictic, hypersaline lake. Microb. Ecol. **47:**30–40.
34. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24:**4876–4882.
35. **Tyrrell, T., E. Maranon, A. J. Poulton, A. R. Bowie, D. S. Harbour, and E. M. S. Woodward.** 2003. Large-scale latitudinal distribution of Trichodesmium spp. in the Atlantic Ocean. J. Plankton Res. **25:**405–416.
36. **Van de Peer, Y.** 2001. TREECON for Windows, version 1.3b. Department of Biology, University of Konstanz, Konstanz, Germany.
37. **Voss, M., P. Croot, K. Lochte, M. Mills, and I. Peeken.** 2004. Patterns of nitrogen fixation along 10°N in the tropical Atlantic. Geophys. Res. Lett. **31**. [Online.] doi:10.1029/2004GL020127.
38. **Wallace, D. W. R., and H. W. Bange.** 2004. Introduction. Results of the Meteor 55: tropical SOLAS expedition. Geophys. Res. Lett. **31.** doi:1029/2004GL021014.
39. **Zani, S., M. T. Mellon, J. L. Collier, and J. P. Zehr.** 2000. Expression of *nifH* genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. Appl. Environ. Microbiol. **66:**3119–3124.
40. **Zehr, J. P.** 1991. Molecular biology of nitrogen fixation in natural populations of marine cyanobacteria, p. 249–264. *In* E. J. Carpenter, D. G. Capone, and J. G. Rueter (ed.), Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs, vol. 362. Kluwer Academic Publishers, Dordrecht, The Netherlands.
41. **Zehr, J. P., E. J. Carpenter, and T. Villareal.** 2000. New perspectives on nitrogen-fixing microorganisms in tropical and subtropical oceans. Trends Microbiol. **8:**68–73.
42. **Zehr, J. P., B. D. Jenkins, S. M. Short, and G. F. Steward.** 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ. Microbiol. **5:**539–554.
43. **Zehr, J. P., M. T. Mellon, and W. D. Hiorns.** 1997. Phylogeny of cyanobacterial *nifH* genes: evolutionary implications and potential applications to natural assemblages. Microbiology **143:**1443–1450.
44. **Zehr, J. P., M. T. Mellon, and S. Zani.** 1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. Appl. Environ. Microbiol. **64:**3444–3450.
45. **Zehr, J. P., J. Waterbury, P. J. Turner, J. P. Montoya, E. Omoregie, G. Steward, A. Hansen, and D. M. Karl.** 2001. Unicellular cyanobacteria fix N2 in the subtropical North Pacific Ocean. Nature **412:**635–638.